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WATER-EXCHANGE IN THE CRAB *HEMIGRAPSUS NUDUS* MEASURED BY USE OF DEUTERIUM AND TRITIUM OXIDES AS TRACERS

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The water-permeability of several decapod crustaceans has been measured by Rudy (1967) using tritiated water (THO) as tracer. Among the animals studied was the Atlantic green crab, *Carcinus maenas*, a good osmotic regulator that has been the subject of many physiological investigations. Smith (1970) restudied *Carcinus maenas*, using deuterium oxide (DHO) as tracer, and made estimates of the water exchange rate and net water influx. Water-exchange rate was found to decrease with the salinity of the medium, as had previously been demonstrated for the very euryhaline crab *Rhithropanopeus harrisi* (Smith, 1967). Smith's results on *Carcinus*, based on DHO, seemed to indicate a markedly higher water-exchange rate than did the results of Rudy (1967), based on THO as tracer. Even after making reasonable corrections for differences in temperature and in the size of experimental animals, there remained a discrepancy in the hourly water-exchange fraction, with the values based on DHO about 20% higher than those based on THO. In this connection it was considered possibly significant that water-exchange values for the prawn *Palaemonetes varians* by Rudy (1967) using THO averaged only half the value obtained by Parry (1955) using DHO. It was further noted that, in unpublished studies, Smith had obtained a comparable 20% discrepancy between the water fluxes calculated from studies on *Rhithropanopeus harrisi*, using DHO and THO as tracers in separate experiments. Taken together, the above three sets of experiments suggest an isotope effect, but no quantitative value should be assigned because of the chain of assumptions involved in the estimates.

In considering the plausibility of an isotope effect between DHO and THO, it is improbable that it could be attributed to differences in diffusion rate among isotopic molecules of water as such. The molecular dimensions of D_2O and H_2O are almost identical (Kavanau, 1964), suggesting similar molecular diffusion rates. Deuterium and tritium oxides in dilute solution are almost entirely in the form of DHO and THO, with molecular weights of 19 and 20, not greatly different from the weight of 18 for H_2O. Wang, Robinson and Edelman (1953) found that the diffusion coefficients of DHO and THO in H_2O did not differ significantly, but that the diffusion coefficient of H_2O^{18} was about 14% higher than the values for DHO and THO. Chinard and Enns (1954) found no difference in the rates

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of passage of DHO and THO across capillary walls in the dog, and Enns and Chinard (1956) likewise found no significant difference in the passage of THO and H_2O^{18} in this experimental situation. Nevertheless, Johnson and Babb find it necessary to observe (1956, page 442), "It is noted that serious disagreement exists among various investigations of the self-diffusion coefficients of water" (involving the use of THO, DHO, and H_2O^{18} as tracers). Paganelli and Solomon (1957), noting the results of Wang *et al.*, (1953), increased their estimates of H_2O fluxes into the red cell, calculated on the basis of THO fluxes, by 14% to compensate for the reported difference in diffusion coefficients. Thus the matter of differences in the diffusion rates of THO, DHO, and H_2O cannot be regarded as settled.

A second possibility for explaining an isotope effect between the uptakes of DHO and THO is that these isotopes might themselves interact with biological membranes in such a way as to lower water-permeability. Kavanau (1964) points out that the structural order and degree of hydrogen bonding are greater in liquid D_2O than in ordinary water, and it may be inferred that the bonding of T_2O is even stronger. But the experiments upon crustaceans cited in the present paper involved the use of D_2O at a concentration of 5%, while T_2O was used at a concentration of only 0.1%, hence it is unlikely that a measurably lower uptake of THO could be attributed to effects of the tritium isotope upon the membranes themselves. Although it may be possible that different species of crustaceans can distinguish between the chemically identical DHO and THO and show different DHO/THO permeability ratios, no evidence for such is known to the authors. It would have been most desirable to restudy *Carcinus,* but this Atlantic species was not available.

If an isotope effect of the magnitude reported is not likely to have resulted either from diffusion rate differences among isotopic water molecules, or as a consequence of alteration of the permeability of the membranes involved, attention should be directed to possible reactions involving the atoms or ions of the hydrogen isotopes themselves. Protium, deuterium, and tritium differ in mass as 1:2:3. The mobility of the ions H^+, D^+, and T^+ in water is regarded as not simply the diffusion of these ions as hydrated particles among water molecules, but as a process of proton transfer (*cf.* Pimentel and McClellan, 1960, page 254). In this process the isotopic ions of D_2O may exchange rapidly between water molecules with the formation of D^+, H^+, OH^-, H_3O^+, and H_2DO^+ ions. The transfer of hydrogen and its isotopes from molecule to molecule may be expected to be mass-dependent and, to the extent that it is involved in the penetration of deuterium and tritium tracers into animals, might account for an observed slower uptake of THO as compared to DHO. In consequence, the DHO tracer would indicate a higher rate of water exchange than is indicated by THO, and the actual exchange of H_2O might be higher than is indicated by the use of either of the heavier isotopic tracers. Until such problems are clarified and a better quantitation made of any possible H_2O/DHO/THO isotope effect, water-permeability values obtained by use of different isotopes of water cannot be directly compared and the possibility must be borne in mind that neither DHO nor THO may indicate the full value of the permeability of animal surfaces to H_2O.

In order to clarify the discrepancy between our results (Rudy 1967, Smith

1970), we have carried out a double-tracer study with DHO and THO. This should eliminate variables of temperature and season as well as of size, sex, and moult stage of the animals used, and should minimize individual operational variation. We hoped that these experiments would allow us to quantify an isotope effect if such existed, or to rule it out as experimental artifact, and to provide an empirical check on the results given by the THO and DHO methods of determining water fluxes as we have used them.

Materials and Methods

This joint study was carried out in late August and early September, 1970, at the Oregon Institute of Marine Biology, University of Oregon, Charleston, Oregon. *Hemigrapsus nudus* (Dana) was selected as the most readily obtainable euryhaline crab, not unlike *Carcinus* in its ecological preferences, and available in a wide size range. Experimental animals were maintained in running seawater (SW) at 12–13° C, or in 8-inch fingerbowls of SW diluted with tap water and kept cool in running SW. The local SW, taken near the mouth of the estuary of the Coos River, had an osmotic concentration of 1009 milliosmoles at the start of the study, dropping with the onset of rains to 960–980 milliosmoles. The osmotic pressures of blood serum and experimental media were determined by use of a Hewlett-Packard Vapor Pressure Osmometer, model 302B, with NaCl standard of 1000 milliosmoles. Blood was collected in amounts of 0.7 to 1 ml with a fine-tipped glass pipette, and discharged into a very small test tube, which was stoppered and placed in boiling water for 10 seconds. The firm white clot which formed was broken up with a slender glass rod, and the sample centrifuged to make the clear serum available. The blood of *T. nudus* clots so strongly that attempts to carry out VPO determinations on whole blood were unsuccessful. Determinations of osmotic pressure of serum as a function of external salinity were carried out on randomly selected crabs not used for the DHO-uptake studies, since the presence of even a small percentage of DHO, as a second volatile solvent, renders VPO determination impossible.

For studies of uptake of DHO and THO, 5% by volume of D_2O (Bio-Rad Laboratories, Richmond, California, 99 moles %) was added to the SW (giving "95% SW"), or to SW diluted with glass-distilled water (DW) so as to approximate an osmotic concentration of 600 milliosmoles ("60% SW"). To each liter of the above "5% D_2O" solutions was added 1 ml of tritiated water (THO) to given an activity of 1 μc per liter.

Exposure of crabs to the above THO/DHO solutions was carried out by one of us (R. I. S.) following as closely as possible the procedure used by Smith (1970) on *Carcinus maenas*. Tests were made in each salinity, at 10° and 20° C. Temperature variation was held within limits of $\pm$ 0.25°. Exposures were for 15 minutes, at the end of which each crab was rinsed quickly in isotope-free medium, wrapped and blotted in absorbent paper, and a blood sample drawn by puncture of the arthrodial membrane at a leg base. A few drops of this sample were immediately placed in the large end of a Pasteur pipette and distilled at 50° C, as in the experiments on *Carcinus* (Smith, 1970) and as described in detail in Welsh, Smith, and Kammer (1968), for determination of DHO. This, and subsequent calcula-

tions of the rate of DHO-uptake as a function of body weight, the determination of the hourly water-exchange fraction (K) on the basis of DHO-uptake, and the determination of Q_{10} for DHO-uptake were carried out by R. I. S. The remainder of the blood sample was discharged into a small screw-capped jar and frozen. These samples were then vacuum-distilled by P. P. R. in a freeze-drying apparatus, as nearly as possible following the method he had used in his study of *Carcinus* (Rudy, 1967), and the THO-saturation (% specific activity) of the blood determined with a Nuclear Chicago scintillation counter. Further treatment of these data was carried out by R. I. S. in order to eliminate any slight methodological differences.

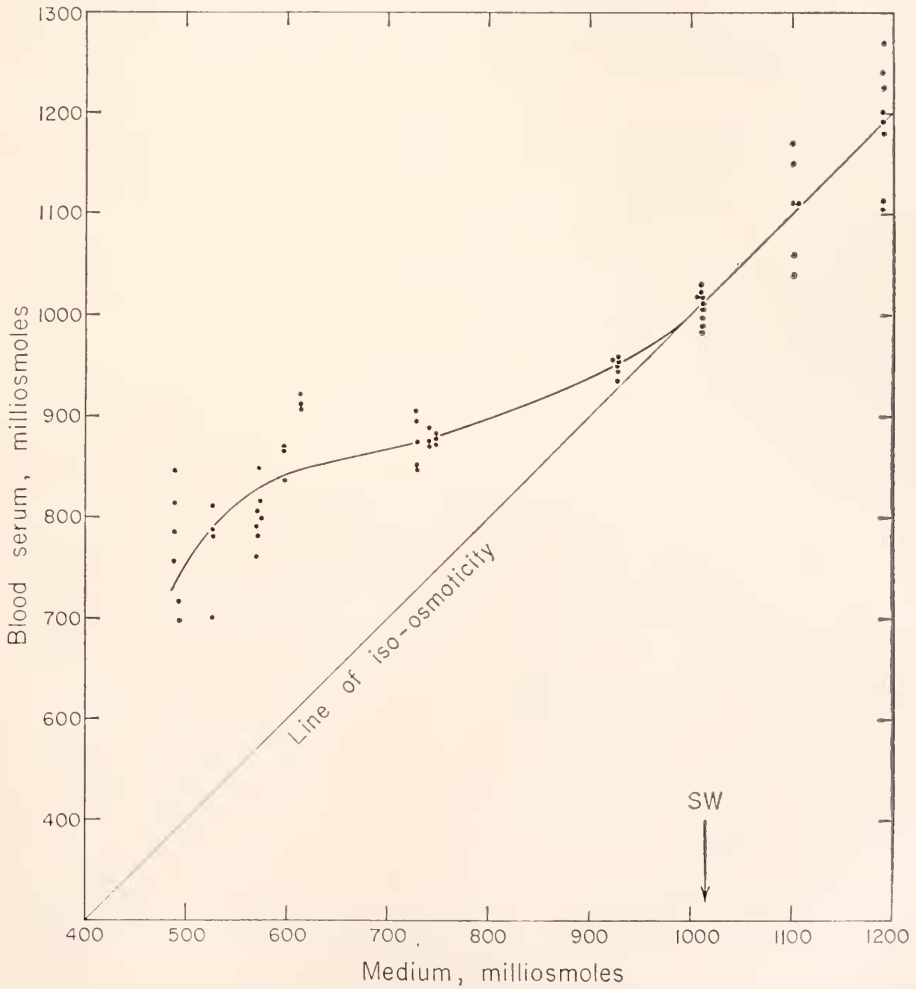

FIGURE 1. Osmotic concentration (milliosmoles) of blood serum of *Hemigrapsus nudus* as a function of concentration of the medium.

Results

(*a.*) *The osmotic pressure of blood serum as a function of salinity*

One of the discrepancies between the results of Rudy (1967) and Smith (1970) regarding water-uptake in *Carcinus* lay in the fact that the authors found different concentrations of chloride in animals adapted to SW, and a relationship between chloride concentration and osmotic pressure of blood had to be assumed on the basis of possibly inappropriate data of earlier authors. VPO determinations of osmotic concentration of serum were agreed upon as a better basis for the estimation of the mole fractions of water in blood of animals adapted in 60% and 95% SW. The results (Fig. 1) show that the blood of *Hemigrapsus nudus* is iso-osmotic to the medium at about 980 milliosmoles (*ca.* 98% SW) under the conditions of the experiments, and hypertonic (*ca.* 865 milliosmoles) to the medium (*ca.* 600 milliosmoles) in 60% SW. From 100% to 120% SW, the blood is iso-osmotic with the medium. Animals in 50% SW showed signs of being over-

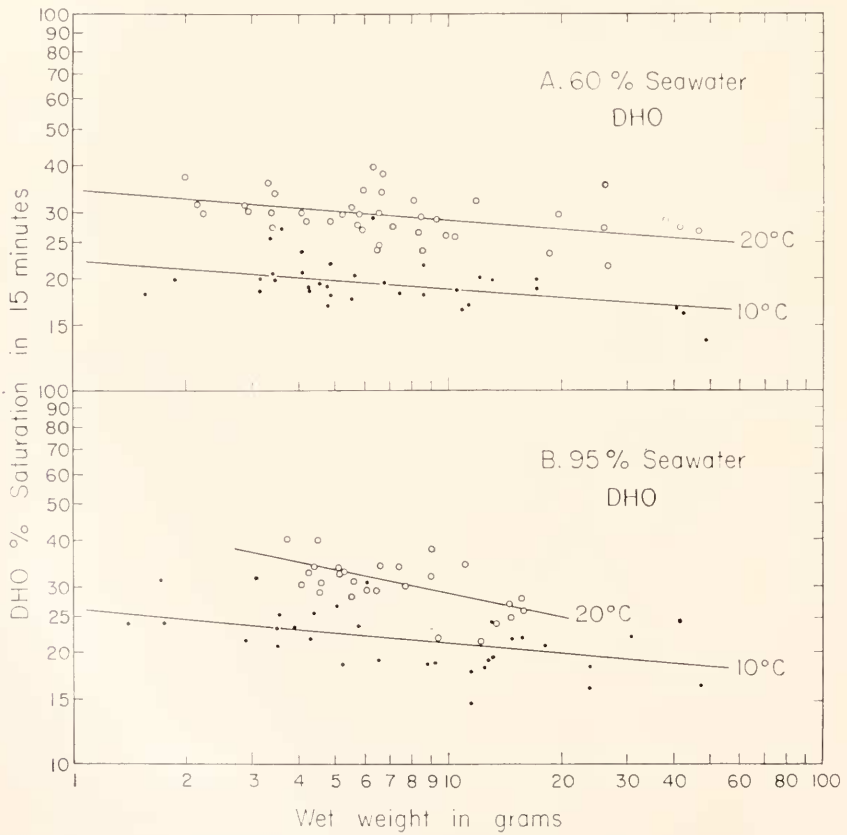

Figure 2. Uptake of DHO by *Hemigrapsus nudus* in a 15-minute exposure, as a function of salinity, temperature, and body weight, expressed as per cent of the concentration of DHO in medium.

stressed, some deaths occurred, and the curve of osmotic regulation showed a noticeable drop below the level of the regulatory plateau. 60% SW, as used in the isotope-uptake tests, appeared to be within the physiologically acceptable regulatory range.

(*b.*) *The uptake of DHO*

The concentration of DHO attained in the blood in a 15-minute exposure in 60% and 95% SW was determined at 10° and 20° C. Animals in three of the groups numbered 34, 41, and 31, with wet weights ranging from less than one to more than 40 grams. Plots of uptake against weight (Fig. 2 and Table I) yielded curves of comparable slopes (b-1 averaging -0.0766). However, in one group, numbering 27 and tested in 95% SW at 20° C, the weight range was only 3.7 to 16 grams. The slope (b-1 $= -0.2091$) is quite different from the rest, and is

TABLE I

Part A: Uptakes of THO and DHO by Hemigrapsus nudus *under four experimental conditions, with probabilities of differences in uptake related to type of isotope being significant ("t" test), calculated for wet weight of 10 g. Part B: Significance of differences in uptake in respect to salinity*

Part A

Experimental conditions		n	Isotope	Mean uptake as % Sat/15 min	Standard deviation	Probability
95% SW	20° C	27	THO	29.92	±3.69	Not significant
			DHO	29.66	±4.16	
	10° C	31	THO	20.93	±3.76	Not significant
			DHO	21.51	±3.46	
60% SW	20° C	41	THO	26.73	±4.34	>0.05
			DHO	28.50	±3.90	
	10° C	34	THO	17.24	±2.68	<0.02
			DHO	18.81	±2.56	

Part B

Salinity	Temp.	n	Isotope	Probability	n	Isotope	Probability
95% SW	20° C	27	DHO	>0.10	27	THO	<0.01
60% SW		41			41		
95% SW	10° C	31		<0.01	31		0.001
60% SW		34			34		

considered unreliable because of the small weight range tested. Therefore, the mean (b-1) value of the other three groups was used in adjusting the uptake values of the small fourth group to 10-g weight. This irregular procedure does not, in fact, change the mean value at 10-g weight very greatly, raising the mean uptake from 28.31% to 29.66% saturation.

When the uptake values of DHO (as % external DHO concentration or "% saturation") attained in 15 minutes are adjusted for a weight of 10 grams, which is not far from the mean weight of the crabs used, the values for DHO uptake are as shown in Figure 5 and Table I. A significant reduction of uptake is shown in 60% SW as compared to 95% SW at 10° C, confirming the apparent reduction of permeability to water (more properly, reduction of water exchange) shown at lower salinities by the crabs *Carcinus* (Smith, 1970) and *Rhithropanopeus* (Smith, 1967). However, the reduction of water-exchange in 60% SW when measured at 20° C is not statistically significant. Probably this results from the in-

TABLE II

Hourly exchange fractions (K) and Q_{10} of DHO and THO uptake by Hemigrapsus nudus, *calculated for a wet weight of 10 g*

Experimental conditions		n	Isotope	Hourly exchange fraction "K"	Q_{10}
95% SW	20° C	27	DHO	1.41	[1.45]
	10° C	31		0.97	
60% SW	20° C	41		1.34	1.61
	10° C	31		0.83	
95% SW	20° C	27	THO	1.42	[1.51]
	10° C	31		0.94	
60% SW	20° C	41		1.24	1.63
	10° C	31		0.76	

adequacy of the sample tested at 20° C in 95% SW. Mean hourly water exchange fractions "K," given by the equation $K = (2.3/t)\ \mathrm{Log}_{10}\ (100/100\text{-}\%\ \mathrm{Sat})$, calculated from the mean DHO uptakes adjusted for a wet weight of 10 g are given in Table II, as are Q_{10} values for DHO uptake estimated by the ratios of "K" at 20° C to "K" at 10° C. Since the sample tested at 20° C in 95% SW is open to question, the Q_{10} value of 1.61 for DHO uptake in 60% SW is considered the more reliable of the two.

(c.) *The uptake of THO*

The concentration of THO attained in the blood in 15-minute exposures in 60% and 95% SW was the result of the same exposures as for DHO. Plots of

uptake against weights (Fig. 3 and Table I) yielded curves with (b-1) values much like those for DHO. One high value (− 0.1486) is regarded as unreliable because of the small weight range of the crabs used in this group (95% SW at 20° C). The average of the remaining three groups was − 0.0705, and this was used in correcting uptake in the deviant group to that of 10-g animals.

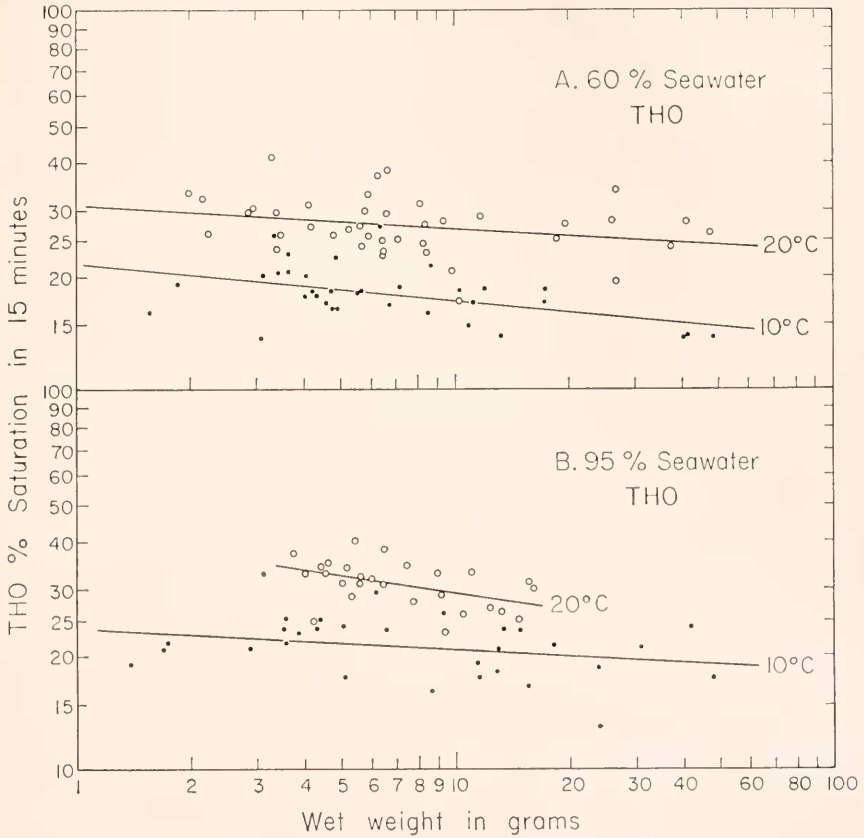

FIGURE 3. Uptake of THO by *H. nudus* in a 15-minute exposure, as a function of salinity, temperature, and body weight.

When the uptake ("% saturation") values attained in 15 minutes are adjusted for a weight of 10 g, the values for THO uptake are as shown in Figure 5 and Table I. A significant reduction of uptake is shown in 60% sea water as compared to 95% sea water in the tests, both at 10° and 20° C.

The mean hourly water exchange fractions (K), adjusted for a wet weight of 10 g are given in Table II, together with Q_{10} values, estimated as before. If we exclude the values from 95% SW because of the questionable validity of the group tested at 20° C, the value of 1.63 may be taken as the Q_{10} of THO uptake. This corresponds well with the value of 1.61 obtained for DHO in 60% SW.

(d.) Comparison of DHO and THO uptake

In three groups out of four, the THO method resulted in lower uptake values than were obtained by the DHO method (Fig. 4, 5). Both methods showed a significant reduction in water exchange at the lower salinity, with the exception of the DHO, 20° C, pair. Using the slopes of the other three groups as representing the actual relationship between salt concentration and water turnover, it would appear that the uptake determined by the DHO method in 95% SW at 20° is out of line and may be rejected.

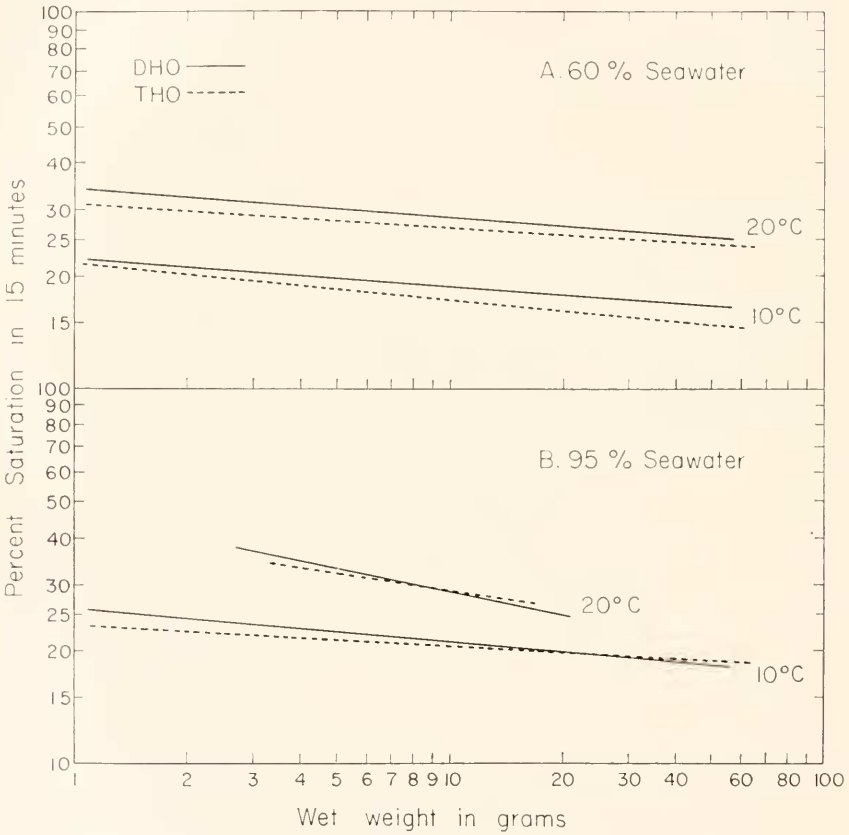

FIGURE 4. Comparison of uptakes of DHO and THO by *H. nudus* in respect to salinity, temperature, and wet weight, to show general smiliarity.

The ratios of K (THO)/K (DHO) derived from the other 3 groups average 0.94, that of the aberrant group being 1.01. The "t" indicates no significant difference between THO and DHO uptakes (as % saturation adjusted to 10 g wet weight) obtained in 95% SW at either 10° or 20° C (Table I). In 60% SW, significance is indicated by a probability greater than 0.05 at 20° C, and less than 0.02 at 10° C. The lack of significance within some groups may reflect low "n" values (41 or less).

However, when the ratio of THO/DHO uptakes is calculated for all paired determinations, involving all 4 groups, totaling 133 animals, the mean uptake of THO is 95.98% that of the DHO uptake. Even including the group tested in 95% SW at 20° C, which was "out of line," this value of 96% is significantly different from 100% at the 1% level of probability (99% confidence limits), and it is concluded that, in our experiments, the measured uptake of THO is significantly lower than that of DHO.

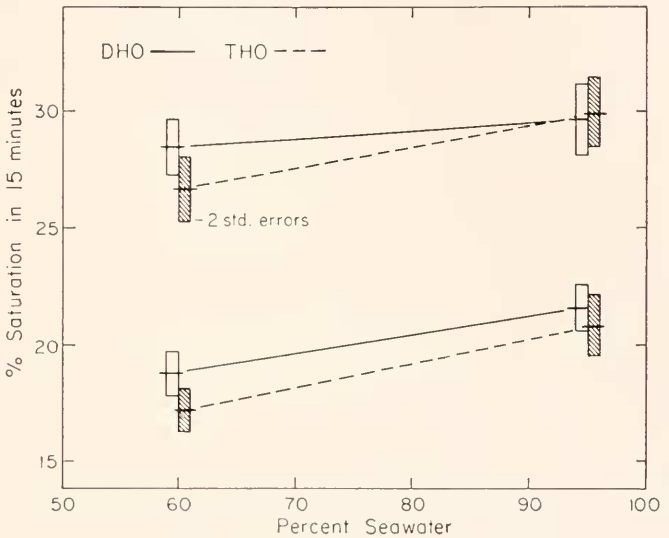

FIGURE 5. Uptake of DHO and THO by *H. nudus* as a function of salinity and temperature, adjusted for a wet weight of 10 grams. Blocks indicate 2 standard errors above and below the means.

DISCUSSION

But the actual meaning of this "significantly lower" uptake of THO in relation to DHO is not so easily determined. That the procedures used gave a small difference is reasonably clear. It is equally clear that the previously-reported difference of 20% (Smith, 1970) is not substantiated. Further refinement of procedures would probably reduce the scatter evident in the present results—in particular, increase of the exposure time so as to achieve saturations of ± 50% would be desirable. The 15-minute exposure was selected on the assumption that *Hemigrapsus nudus* would have a permeability comparable to that of *Carcinus,* but it proved to be less permeable than expected (*Carcinus:* K (DHO) = 2.07 in 50% SW at 18° C, Smith 1970; *H. nudus:* K (DHO) = 1.34 in 60% SW at 20°, this study).

It is possible that the difference observed between DHO and THO uptake lay in some aspect of the distillation procedures: possibly giving a spuriously high value for DHO expressing itself in the density determinations, possibly giving a spurious low value for THO as a result of undetected contamination (dilution) with atmospheric water in the vacuum distillation.

An attempt was made to make a second determination of DHO upon the vacuum-distilled samples, but this was unfortunately delayed for some weeks and, because many of these samples were contaminated in such a way as to raise their density, the results had to be discarded. The evidence for an isotope effect is not conclusive. We can conclude that the methods of the respective authors for DHO and THO determinations have yielded differing results suggestive of an isotope effect. But while it may be expected that adherence to a single method of distillation may make our procedures more alike, the fact that different final steps must be used to determine concentrations of the radio-active THO and the non-radio-active DHO makes conclusive demonstration of an isotope effect unattainable by our methods.

Despite this difficulty, we have in fact obtained different measures of water flux on the same experimental animal and, if we accept the possibility of an isotope effect, it follows that the actual flux of H_2O may be higher than that indicated by heavier isotopes. If a mass difference of 3:2 (T:D) results in fluxes in the proportions of 96:100, then a mass difference of 2:1 (D:H) might be expected to produce a difference in fluxes as great as 100:105.3. In other words, the relative uptakes of THO, DHO, and H_2O might be as *ca.* 91:95:100.

These differences in uptake as indicated by different isotopic tracers are not great, but may partly explain why measured values of urine production in crabs have in some cases been greater than those predicted from water-exchange measurements, as, for example, the urine volumes measured in *Carcinus* in 30% SW by Shaw (1961) and Binns (1969), as compared to the net influx values calculated on the basis of THO exchange (Rudy, 1967) and DHO exchange (Smith, 1970). In Table III (line 11) is shown a calculation of the daily net influxes of water at 20° C (presumed equal to urine volume) in *Hemigrapsus nudus*, calculated on the above assumption that uptakes of THO:DHO:H_2O are in the proportions of 91:95:100. These values are less than comparable values reported for *Carcinus* (line 12), indicating that *Hemigrapsus nudus* has a lower net water influx and should produce less urine than *Carcinus*. The values for net water influx in *Carcinus* in 60% SW as previously determined by Smith (DHO) and Rudy (THO) are caluculated and shown in line 12 (Table III), together with urine volume as estimated by H_2O-clearance methods by Shaw (1961). These values show an overall inverse relation to isotope weight consistent with the present hypothesis of an isotope effect. This trend is less clear in the case of 95% SW, but it may be noted that the net influx value from Rudy (THO) and urine volume from Shaw (clearance) had to be obtained by interpolation. The results are only consistent with the concept of an isotope effect; they do not prove it. What they show is that the THO method for some reason tends to indicate a low value for water flux relative to the DHO method, and that measures of urine volume in crabs by methods not involving the heavier isotopes of water may yield higher values. However, the difference between urine volumes as determind by methods not involving water isotopes, and the net influxes calculated from water-isotope exchange studies, is still too great to be explained solely on the basis of an isotope effect as small as the 5–10% suggested here. Such factors as unstirred layers or pores may also be involved. And, obviously, studies by several methods used upon the same species should furnish more satisfactory data than is currently available.

TABLE III

(Lines 1–11) Calculation of water influxes in Hemigrapsus nudus *at 10° C in 60% and 95% SW according to hourly water exchange (K) values based upon simultaneous THO and DHO uptake, with estimates of net H_2O influx. Water content assumed to be 70%.*
(Line 12) Published comparable values for Carcinus

1. Medium, % SW and conc. millios-moles	60 (600 millismoles)			95 (950 millismoles)		
2. Concentration of blood, millios-moles	845			965		
3. Conc. of blood as % SW	84.5			96.5		
4. Mole fraction water of medium	0.9893			0.9832		
5. Mole fraction water of blood	0.9850			0.9829		
6. Mole fraction difference	0.0043			0.0003		
7. Hourly water exchange fraction (K) at 10° C	THO	DHO	H_2O (est.)	THO	DHO	H_2O (est.)
	0.76	0.83	*ca.* 0.855	0.94	0.97	*ca.* 1.025
8. Daily water influx (K × 70 × 24) as % body weight per day	1276.8	1394.4	1436.4	1579.2	1629.6	1722.0
9. Daily *net* water influx as % of total influx	0.435	0.435	0.435	0.0305	0.0305	0.0305
10. Daily *net* influx as % of body weight at 10° C	5.55	6.06	6.25	0.48	0.50	0.525
11. Daily net influx in *H. nudus* at 20° C (Q_{10} = 1.62)	8.99	9.82	10.1	0.78	0.81	0.85
12. Calculated daily net influx in *Carcinus* (DHO and THO) and ¶ urine volume (direct meas.)	net influx 9.4 Rudy (1967) recalc. 20°	net influx 14.5 Smith (1970) recalc. 18°	urine vol. 16.5 Shaw (1961) 16°	net influx 1.25 Rudy (1967) recalc. 20°	net influx 1.19 Smith (1970) recalc. 18°	urine vol. 5.0 Shaw (1961) 16°

SUMMARY

1. The crab *Hemigrapsus nudus* regulates the osmotic pressure of its blood in media down to less than 60% seawater, and is iso-osmotic in 100% seawater and higher salinities.

2. Measurements of simultaneous uptake of tritiated water (THO) and deuterated water (DHO) give uptake values for THO about 96% those obtained with DHO.

3. The Q_{10} of uptake of both isotopes is about 1.62, and the relation of uptake of both to body weight is similar.

4. The results are consistent with, but do not prove, the concept of a small isotope effect in the uptake of THO and DHO. Published reports of higher water fluxes based on methods not involving isotopes of water are consistent with the argument for an isotope effect. It is suggested that water fluxes based on methods using THO:DHO:H_2O as ordinarily employed are of the relative magnitudes 91:95:100.

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